Mutational Analysis of the Tobacco Vein Mottling Virus Genome

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We have used a cDNA clone of the potyvirus, tobacco vein mottling virus, to construct 19 mutants bearing 12-nt insertions in the viral genome. These mutants display a variety of phenotypes in inoculated tobacco plants or protoplasts. All mutants with insertions in P3, CI, 6K, NIa, or NIb failed to produce detectable amounts of progeny viral RNA in protoplasts or plants which suggests that they all may be directly involved in replication. Mutants (one in P1 and one in HCpro) presumably affected in polyprotein processing also did not replicate in plants or protoplasts. Seven mutants, with insertions in the 5' noncoding region, P1, HCpro, or CP regions of the genome, were able to infect protoplasts. Three of the 7 mutants (1 in the 5' noncoding region and 2 in HCpro) were able to infect protoplasts but not plants. The remaining 4 mutants replicated in protoplasts and were able to cause systemic infection in plants. The mutation in the CP had no effect on virus accumulation or symptom development in inoculated plants, whereas the other 3 (1 in P1 and 2 in HCpro) induced cyclical patterns of symptom expression. These symptoms ranged from very mild to wild-type-like as new leaves emerged and, as the plants continued to grow, this pattern was repeated. These results support the assignment of roles in replication to five coding regions in the genome and demonstrate that sequence alterations in many parts of other regions of the viral genome may have pronounced effects on replication and the expression of disease symptoms. © 1994 Academic Press, Inc.

INTRODUCTION

The potyviruses are a large group of plant viruses with single-stranded, positive-sense RNA genomes of approximately 10 kb and are included in the picornavirus-like supergroup of viruses. A potyviral genome map has been established and the general scheme by which coding regions within the viral RNA are expressed has been determined (reviewed by Riechmann et al., 1992). The potyviral genome encodes a large polyprotein that is proteolytically processed to yield the coat protein (CP) and six or more nonstructural proteins.

Over the years, biochemical analyses of the functions of some potyviral proteins have been reported, and most of these proteins have been shown to be multifunctional. Helper component (HCpro) was one of the first to be analyzed and was shown to be required for transmission of the virus by aphids (Pirone and Thornbury, 1983). The HCpro (Carrington et al., 1989), P1 (Verchot et al., 1991), and NIa (Carrington and Dougherty, 1987) proteins have been shown to have proteinase activity and to be involved in processing of the viral polyprotein. The CI protein has in vitro RNA-dependent ATPase and helicase activities (Laín et al., 1990, 1991). P1 has also been shown to have RNA-binding properties (Brantley

and Hunt, 1993). CP is involved in encapsidation of the viral RNA and also contains a sequence motif that is a determinant of aphid transmissibility (Atreya et al., 1990). NIa functions as a serine-type (trypsin-like) proteinase responsible for six of the polyprotein cleavages (Carrington and Dougherty, 1987; Carrington et al., 1988) and as the protein (VPg) covalently linked to the 5' end of the viral RNA (Shahabuddin et al., 1988; Murphy et al., 1990).

In contrast, functions of other virus-encoded proteins are unknown or have been inferred from amino acid sequence comparisons with other viral proteins. NIb is believed to be the RNA-dependent RNA polymerase based on computer sequence comparisons with other viral polymerases (Koonin, 1991), but polymerase activity has not been demonstrated. The P3 protein has been observed in infected cells (Rodríguez-Cerezo and Shaw, 1991), but its function remains unknown.

A genetic approach examining the function(s) of the potyvirus-encoded proteins would complement the in vitro studies and provide insights into the roles of the proteins in replication and pathogenicity in infected plants. In the present study, we have used a full-length cDNA clone of tobacco vein mottling virus (TVMV) to construct a series of linker insertion mutations at known locations throughout the TVMV genome. The mutants were analyzed by inoculation of tobacco protoplasts and plants with transcripts derived from mutant cDNA clones. The effects of the mutations on replication of
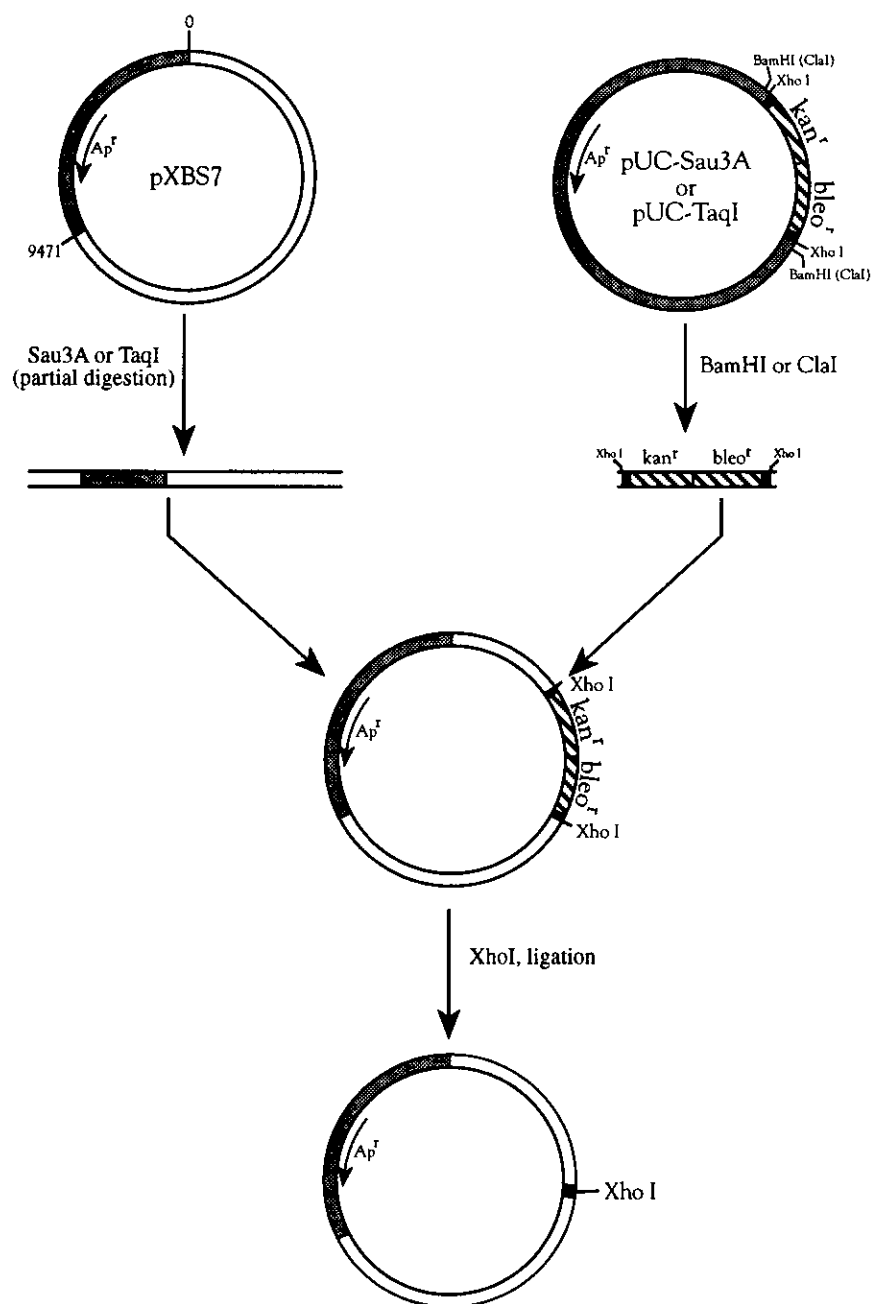
FIG. 1. Strategy for the construction of 12-nt insertions into *Sau*3A or *Taq*I sites in pXBS7, a plasmid containing a full-length DNA copy of TVMV RNA. The open boxes indicate regions of the plasmids corresponding to TVMV sequences and the stipled areas depict plasmid sequences. The kan[r] and bleo[r] genes are shown as a hatched box. The 12-nt sequences inserted into the TVMV polyprotein are depicted by a solid box.

viral RNA and systemic disease development will be described.

MATERIALS AND METHODS

Construction of mutants

Insertion mutations throughout the TVMV genome were generated by cloning a mutagenesis cassette into full-length TVMV cDNA that had been partially digested with *Sau*3A or *Taq*I (in pXBS7; Domier *et al.,* 1989, and Fig. 1). To assemble the mutagenesis cassette the multi-

ple cloning sites in pUC18 were replaced with the sequences AATTGGATCC*CTCGAG*GGATCCAGCT or AAT-TCATCGAT*CTCGAG*ATCGATGAGCT using appropriately designed oligonucleotides to yield pUC-*Sau*3A or pUC-*Taq*I, respectively. Underlined nucleotides designate *Xho*I sites; nucleotides in bold type constitute *Bam*HI and *Cla*I sites in pUC-*Sau*3A and pUC-*Taq*I, respectively. Next, the 1.6-kbp kan[r]–bleo[r] cassette from the plasmid pKIXX (Pharmacia, Piscataway, NJ) was inserted as an *Xho*I fragment into pUC-*Sau*3A and pUC-*Taq*I plasmids. The cassette was then excised with *Bam*HI (for

pUC-Sau3A) or ClaI (for pUC-TaqI) and cloned into the partially digested TVMV cDNA as described below.

For the construction of the Sau3A insertion mutants, 3 μg of TVMV cDNA clone, pXBS7, were partially digested with 0.01 or 0.02 units of Sau3A for 30 min at 37°. The reactions were terminated by heating to 65° for 10 min. Linearization of the plasmid was monitored by electrophoresis in 1% agarose gels. Visualization of the ethidium-bromide-stained gel revealed that approximately 5% of the plasmid was linearized and the remainder uncut. Samples were extracted with phenol/chloroform and precipitated with ethanol. The partially digested cDNA was ligated to the 1.6-kbp BamHI mutagenesis cassette containing the kan^r–bleo^r genes flanked by XhoI sites. After transformation of Escherichia coli strain NM522, recombinants were selected by plating on media containing carbenicillin and kanamycin. Recombinant plasmids were isolated and insertion mutants were identified by digestion with XhoI. Since the full-length TVMV cDNA clone does not contain an XhoI site, only recombinant plasmids containing the mutagenesis cartridge could be digested with XhoI. To remove the kan^r–bleo^r gene cassette, selected clones were digested with XhoI and religated. Following transformation and plating on media containing carbenicillin, transformants containing the 12-nt insertion were identified by replica plating on media containing carbenicillin or carbenicillin plus kanamycin. The resulting plasmid retained a 12-nt insertion sequence (CCTCGAGGGATC) in a Sau3A site. The site of the insertion in the plasmids was determined by restriction enzyme analysis and confirmed by dideoxynucleotide sequencing (Hsiao, 1991).

A similar collection of insertion mutants containing a second 12-nt insertion (TCTCGAGATCGA) was created from plasmid pXBS7 linearized by partial digestion with TaqI. Three micrograms of pXBS7 DNA were digested with 0.05 units of TaqI for 30 min at 65°. The partially digested cDNA was ligated to the 1.6-kbp ClaI mutagenesis cassette containing the kan^r and bleo^r genes flanked by XhoI sites and transformed into E. coli strain GM2929. Identification of the site of insertion and the removal of the kan^r and bleo^r genes to create a 12-nt insertion was accomplished as described above.

To ensure that frameshift mutations were not introduced into the TVMV cDNA during mutant construction, transcripts of the mutant plasmids (Rodríguez-Cerezo et al., 1991) were translated in vitro in the rabbit reticulocyte lysate and wheat germ extract systems (Promega, Madison, WI) and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The pattern of proteins synthesized was compared to the pattern synthesized from native TVMV RNA.

Inoculation of protoplasts and plants with mutant transcripts

The in vitro synthesis of full-length TVMV RNA was performed as previously described (Rodríguez-Cerezo et al., 1991). Nicotiana tabacum L. (cv Xanthi) mesophyll protoplasts were prepared and inoculated by electroporation as described by Luciano et al. (1987) using a Bio-Rad Gene-Pulser. Typically, 1.5 × 10^6 protoplasts were electroporated with 100 μl of in vitro transcription reaction mixture. After electroporation, the protoplasts were placed in incubation buffer (Takebe et al., 1968) at a concentration of 2 × 10^5 protoplasts/ml and incubated at 23°. Samples (1 × 10^5 protoplasts), in which the accumulation of CP was to be measured, were harvested after incubation for 48 hr and extracts were analyzed as described below. For measurements of the accumulation of progeny viral RNA, protoplasts (2 × 10^5) were harvested after incubation for 24 and 48 hr. Analysis of protoplasts incubated for the two periods provided a means of distinguishing between progeny viral RNA and the residual inoculum transcript RNA. The activity of each mutant in inoculated protoplasts was assessed at least four times and the results presented are those of a typical experiment.

To inoculate N. tabacum L. cv Kentucky 14 plants, 2 vol of in vitro transcription reaction mixture were mixed with 1 vol of inoculation buffer [110 mM sodium pyrophosphate, pH 9.0, 3 mM EDTA, and 3% (w/v) bentonite]. Two leaves (positions 2 and 3, denoted here by their numerical location above the oldest leaf on the plant at the time of inoculation) of each plant were mechanically inoculated (7.5 μl/leaf) using Carborundum as an abrasive. Control plants were mock-inoculated or inoculated with native TVMV RNA at a concentration of 33 μg/ml.

Detection of infection in inoculated plants

Plants were tested for the incidence of infection beginning approximately 14 days after inoculation and continuing every 3–4 days for the next 4–6 weeks. Three 6-mm discs were taken from leaves of each plant and the amount of TVMV CP in these samples was determined by direct double-antibody sandwich ELISA (Clark and Adams, 1977). The leaf discs were sonicated in 300 μl of sample buffer (10 mM phosphate buffer, pH 7.4, 120 mM NaCl, 0.05% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin). After sonication, 200 μl sample buffer was added, the samples were centrifuged in an Eppendorf microcentrifuge for 5 min and 1:1, 1:10, and 1:30 dilutions of the supernatants in sample buffer were then processed for direct ELISA using anti-TVMV CP serum. Leaves 6 and 15 (three 12-mm discs) of each inoculated plant were also analyzed for the presence of viral RNA as described below.

Western blot analysis of inoculated protoplasts

Samples (1 × 10^5 protoplasts) were taken 48 hr after inoculation and sedimented in a microcentrifuge at 1000 g for 3 min. The protoplast pellets were resuspended in 25 μl 4× disruption buffer (1× = 62 mM Tris–HCl, pH

6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol), boiled for 5 min, and centrifuged for 3 min in a microcentrifuge. Proteins in the extracts were separated by electrophoresis in 10% polyacrylamide gels (Laemmli, 1970) and transferred to Immobilon P membranes (Millipore, Bedford, MA) (Towbin et al., 1979). Following transfer, the presence of TVMV CP was determined by immunoblot analysis as described by Domier et al. (1989). Blots were scanned with an image acquisition densitometer (Biolmage, Milligen/Biosearch, Ann Arbor, MI) to determine the relative staining intensity of the immunologically reactive protein and quantified on the basis of whole-band analysis.

Extraction and analysis of RNA

Total nucleic acids were extracted from protoplasts and leaf tissue as described by Klein et al. (1988) with minor modifications. Samples (2 × 10^5 protoplasts or 0.1 g ground frozen leaf tissue) were resuspended in 300 μl RNA extraction buffer (100 mM Tris–HCl, pH 9.0, 300 mM NaCl, 20 mM EDTA, 2% SDS, 250 μg/ml proteinase K, 500 μg/ml heparin), incubated for 20 min at 37°, and extracted with phenol/chloroform. The organic phase of each sample was extracted twice with RNA extraction buffer and the nucleic acid precipitated with ethanol overnight at −80°. After centrifugation for 20 min at 4°, the samples were washed and resuspended in RNA solubilization buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1% SDS). LiCl was added to 2 M and RNA allowed to precipitate overnight at 4°. The RNA was collected by centrifugation and dissolved directly in formaldehyde/gel loading buffer (Sambrook et al., 1989).

Northern blot analysis of RNA electrophoresed in 0.8% agarose–formaldehyde gels was as described by Sambrook et al. (1989). RNA was transferred to GeneScreen membranes (New England Nuclear, Wilmington, DE) by the capillary blot procedure. Viral RNA was detected with a ^{32}P-labeled complementary RNA probe (nucleotides 3872–5845 of TVMV RNA) prepared by in vitro transcription of a subclone of pXBS7 (Rodríguez-Cerezo et al., 1991). To determine the relative ^{32}P incorporation, autoradiograms were scanned and quantified as described above.

Analysis of progeny viral RNA from infected plants

RNA isolated from plants that became infected after inoculation with transcript RNA was tested for the presence of the mutation in the progeny viral RNA. Total RNA was isolated from plants 15–20 days (leaf 6) and 45 days (leaf 15) after inoculation as described above. Following reverse transcription and polymerase chain reaction amplification (RT-PCR), the products were analyzed by restriction enzyme digestion. For RT-PCR, first strand cDNA was produced from 1 μg total RNA in a 20-μl reaction volume using 5 units of avian myeloblastosis virus re-

verse transcriptase (Life Sciences, St. Petersburg, FL) and 2.5 pmol of a minus-sense primer (i.e., binds to DNA of the same sequence as viral RNA; 17 to 24 nt) specific for the region of the viral genome near the particular 12-nt inserted sequence. After incubation for 1 hr at 42°, the enzyme was inactivated by heating to 95° for 5 min. For PCR amplification, a 100-μl reaction mixture contained 20 μl of first strand cDNA, 10 μl of 10× PCR buffer I (Perkin–Elmer, Norwalk, CT), 0.2 mM dNTPs, 50 pmol of a specific plus-sense primer (i.e., binds to DNA complementary to viral RNA; 17 to 24 nt), 47.5 pmol of the minus-sense primer, and 2.5 units Taq polymerase. Amplification conditions were 94°, 1 min 20 sec; 45°, 2 min; 72°, 3 min for 30 cycles. The PCR products were extracted with phenol/chloroform and precipitated with ethanol. The presence or absence of the mutation in the progeny viral RNA was examined by digestion of the purified RT-PCR samples with XhoI. In addition, RT-PCR products from two mutants were digested with HindIII and the 0.99-kbp HindIII fragments containing the region of the mutation were gel-purified (GeneClean protocol, Bio101, La Jolla, CA) and cloned into pKS+ phagemid (Stratagene, La Jolla, CA). The region in the plasmid DNA surrounding the mutant insertion was subsequently sequenced (Hsiao, 1991).

RESULTS

Construction and mapping of mutations

To investigate the roles of individual virus-encoded proteins in replication and pathogenicity, linker insertion mutations were constructed throughout the TVMV genome. This resulted in a series of mutants each of which contained a 12-nt insertion at a Sau3A or TaqI site in the genome. The inserted sequences were designed to introduce a unique XhoI site into the TVMV cDNA clone to facilitate mapping and to aid in the screening of progeny viral RNA. With this strategy, a collection of 19 mutants was obtained (Table 1 and Fig. 2). Thirteen mutants contained the sequence CCTCGAGGGATC at Sau3A sites and resulted in insertion of one of three 4-amino acid segments into the viral polyprotein (Table 1). One additional Sau3A insertion mutant in the 5′ noncoding region was obtained. Five mutants containing the sequence TCTCGAGATCGA at selected TaqI sites were also generated and resulted in the addition of one of two 4-amino acid segments into the polyprotein (Table 1). With two of the mutants, one in the 5′ noncoding region (5′UTR-151Δ19) and one in HCpro (HC-1937Δ15), a small deletion of TVMV sequence occurred at the site of the 12-nt insertion as a result of cleavage of pXBS7 at two closely spaced Sau3A sites. Mutant designations refer to the coding region and the nucleotide residue positions at which the insertions occurred. The names of the two mutants containing small deletions of TVMV

TABLE 1

NAMES OF TVMV MUTANTS AND LOCATIONS OF INSERTIONS IN VIRAL RNA

| Mutant | Location of insert | | Amino acid sequence[b] |
	nt[a]	Gene	
5'UTR-151Δ19[c]	151	5'UTR	
P1-616	616	P1	Arg(137)−Ser−Arg−Asp−Arg−Val(138)
P1-801	801	P1	Arg(198)−Ser−Leu−Glu−Gly−Ser(199)
HC-1378	1378	HCpro	Arg(391)−Ser−Arg−Asp−Arg−Leu(392)
HC-1500	1500	HCpro	Gly(431)−Ser−Leu−Glu−Gly−Ser(432)
HC-1937	1937	HCpro	Asp(577)−Pro−Ser−Arg−Asp−Pro(578)
HC-1937Δ15[c]	1937	HCpro	Asp(577)−Pro−Ser−Arg−Asp−Asp(582)
HC-2201	2201	HCpro	Asp(665)−Pro−Ser−Arg−Asp−His(666)
P3-2514	2514	P3	Leu(769)−Asp−Leu−Glu−Ile−Glu(770)
P3-3208	3208	P3	Ile(1001)−Pro−Arg−Gly−Ile−Lys(1002)
P3-3322	3322	P3	Arg(1039)−Ser−Arg−Asp−Arg−Gln(1040)
CI-4040	4040	CI	Asp(1278)−Pro−Ser−Arg−Asp−Arg(1279)
CI-4411	4411	CI	Ile(1402)−Pro−Arg−Gly−Ile−Ile(1403)
CI-4467	4467	CI	Ile(1420)−Asp−Leu−Glu−Ile−Glu(1421)
CI-5059	5059	CI	Ile(1618)−Pro−Arg−Gly−Ile−Trp(1619)
6K-5516	5516	6K	Asp(1770)−Pro−Ser−Arg−Asp−Leu(1771)
NIa-6652	6652	NIa	Ile(2149)−Pro−Arg−Gly−Ile−His(2150)
NIb-7386	7386	NIb	Gly(2393)−Ser−Leu−Glu−Gly−Ser(2394)
CP-8465	8465	CP	Asp(2753)−Pro−Ser−Arg−Asp−Gln(2754)

[a] The insertion occurs after the indicated nucleotide (nt) in TVMV RNA.

[b] Inserted amino acids are in bold type; numbers are positions of amino acid residues in the TVMV polyprotein.

[c] During mutant construction, deletion of a small fragment of the TVMV cDNA occurred at the site of the insertion; the name of the mutant includes the number of nucleotides deleted (Δ15 or Δ19).

sequence at the site of the 12-nt insertion also include the number of nucleotides deleted (i.e., Δ15 or Δ19).

To ensure that a frameshift had not occurred in any of the mutants, RNA was synthesized and translated *in vitro* and the products analyzed by SDS–polyacrylamide gel electrophoresis. In none of the mutants was there evidence of an interruption of the reading frame. In most cases, the pattern of translation products obtained with each mutant was identical to that seen with pXBS7 RNA (data not shown). However, some of the mutants that

contained insertions in regions thought to be responsible for polyprotein processing (P1-616, HC-2201, and NIa-6652) did produce altered patterns of protein synthesis. At present, it is unclear to what extent these three mutants are affected in proteolytic processing.

Effects of mutations on accumulation of viral RNA in protoplasts

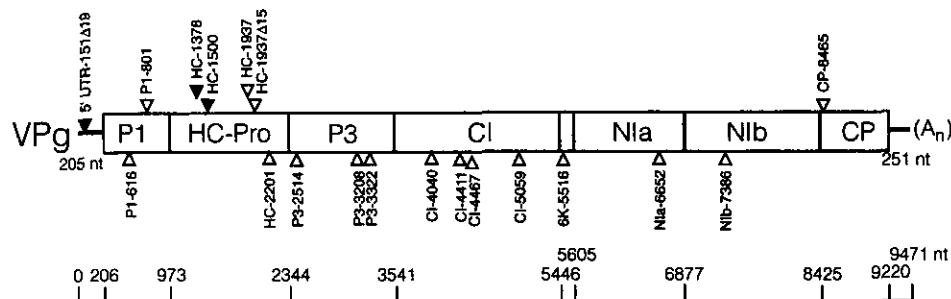The effects of the insertions in the TVMV genome on the infectivity of viral RNA were examined by inoculating

FIG. 2. Locations and phenotypes of insertion mutations in the TVMV genome. The linear array of open boxes depicts the individual proteins that result from proteolytic processing of the TVMV polyprotein. Names of proteins are within boxes. Untranslated regions are shown as horizontal lines at each end of the map. The positions of the insertion mutations and their phenotypes are marked by triangles. The phenotypes of individual mutants were determined by immunoblot and Northern analyses of proteins and RNA, respectively, from inoculated tobacco protoplasts incubated for 48 hr and by direct double-antibody sandwich ELISA of leaf tissue from inoculated plants harvested 2 weeks after inoculation. Triangles below the map represent lethal mutants. Triangles above the map represent viable mutants: open triangles above the map indicate mutants that replicate in both protoplasts and plants, and closed triangles indicate mutants that replicate in protoplasts but not in plants. The scale beneath the genome map indicates nucleotide residue positions where coding regions begin and end.
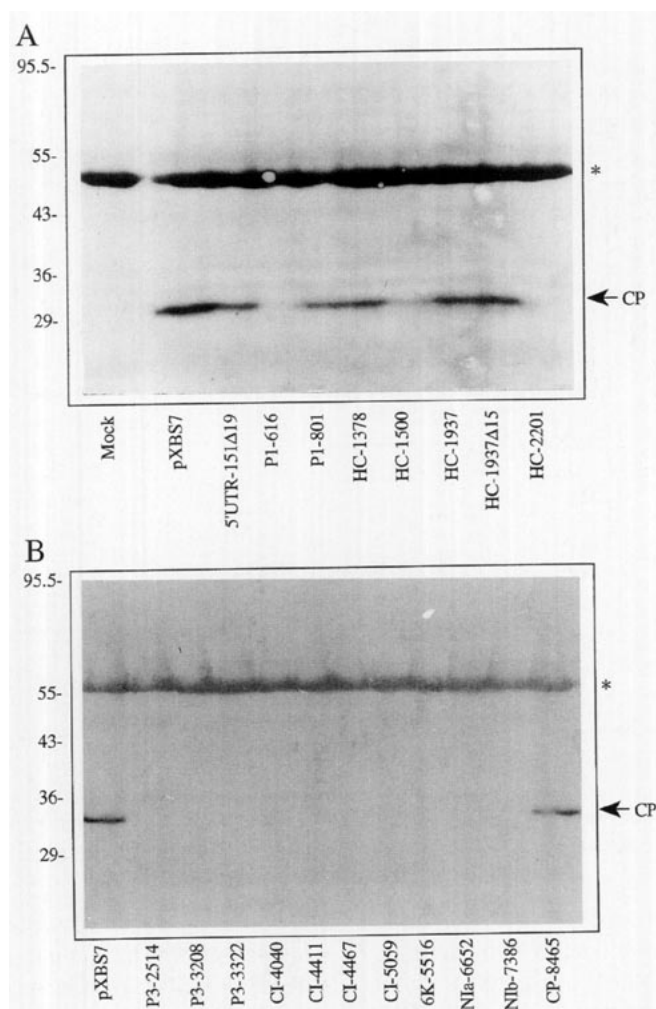
FIG. 3. Immunoblot analysis of viral coat protein accumulation in tobacco protoplasts inoculated with TVMV mutants. Protoplasts were either mock-inoculated (Mock) or inoculated with transcript RNA synthesized from wild-type plasmid (pXBS7) or from mutant plasmids (names below gel lanes). Protoplasts were sampled 48 hr after inoculation and proteins were electrophoresed in SDS-containing polyacrylamide gels followed by electrophoretic transfer to Immobilon P membranes. Protein blots were treated with antisera directed against TVMV CP. Arrow at the right marks the position of TVMV CP. The antisera also reacted with the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase which is marked with an asterisk. Numbers to the left of the blots indicate the mobility of molecular mass standards (kilodaltons). Immunoblots in A and B are from separate protoplast experiments.

protoplasts with *in vitro*-synthesized mutant transcripts. Twelve of the mutants failed to produce detectable amounts of viral CP or RNA in inoculated protoplasts (Figs. 3 and 4). These included all of the mutations in the P3, CI, 6K, NIa, or NIb coding regions. In addition, progeny viral RNA was not detected in protoplasts inoculated with one mutant in P1 (P1-616) and one in HCpro (HC-2201). In contrast, seven mutants were able to infect protoplasts, although the extent of accumulation of CP and RNA was variable. Scanning of the original protein blots and autoradiograms revealed that protoplasts inoculated with the 5' noncoding region mutant (5'UTR151Δ19) or with mutants P1-801, HC-1378, HC-1937, or HC-1937Δ15 accumulated levels of CP and viral RNA that were 35–60% of the levels observed in protoplasts inoculated with wild-type pXBS7 RNA (Figs. 3A and

4A). The accumulation of CP and viral RNA in protoplasts inoculated with RNA derived from mutant HC-1500 was approximately 15% of wild-type levels (Figs. 3A and 4A). Only one mutant, CP-8465, produced wild-type levels of CP and viral RNA in protoplasts (Figs. 3B and 4B).

Effects of mutations on symptom expression in inoculated plants

The 19 insertion mutants were screened for their ability to multiply and spread in plants. Leaves of small tobacco plants were mechanically inoculated with aliquots of the *in vitro* transcription mixtures from each mutant. The 12 mutants that failed to produce detectable amounts of viral RNA or CP in protoplasts (Figs. 3 and 4) were unable to cause systemic infections in tobacco plants. In none of the plants inoculated with these mutants did CP accu-
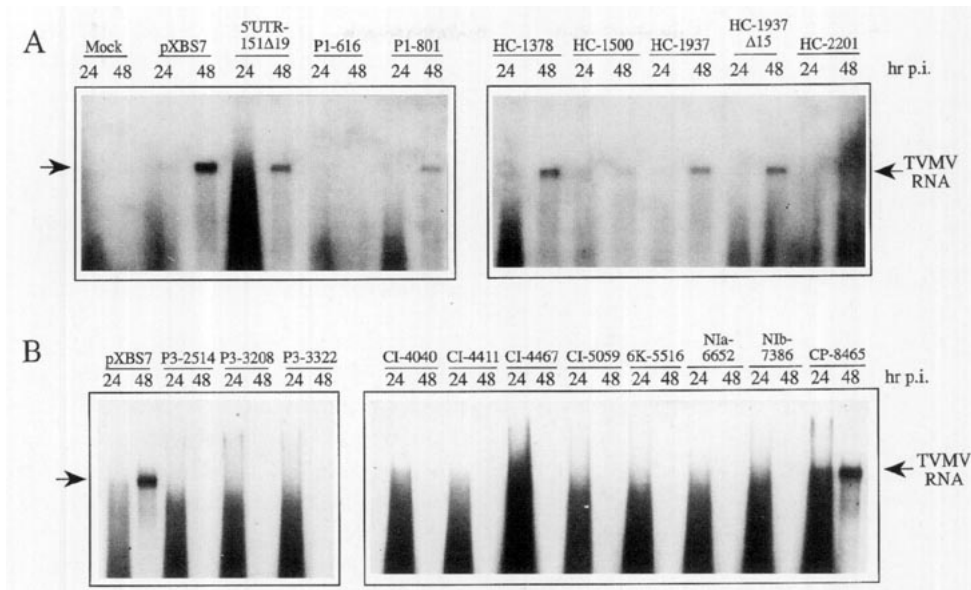
FIG. 4. Northern blot analysis of progeny TVMV RNA accumulation in protoplasts inoculated with mutant-derived RNA transcripts. Protoplasts were either mock-inoculated (Mock) or inoculated with transcript RNA synthesized from wild-type plasmid (pXBS7) or from individual mutant plasmids (names above gel lanes). Protoplasts were sampled 24 and 48 hr after inoculation as indicated above the lanes. Total RNA was extracted from protoplasts, separated on 0.8% agarose–formaldehyde gels, transferred to nylon membranes, and hybridized with a TVMV cRNA probe. The arrow marks the position of TVMV genomic RNA. Blots in A and B were exposed to X-ray film for 5 and 4 days, respectively. Shorter exposure of the blot in A showed no positive hybridization in the 48-hr HC-2201 sample.

mulate to detectable levels or were disease symptoms observed. Inoculation of plants with 3 other mutants (5′UTR-151Δ19, HC-1378, and HC-1500) also failed to result in systemic infections despite their replication in isolated protoplasts. Neither disease symptoms nor accumulation of CP was detected in plants inoculated with these mutant RNAs.

Of the seven mutants that infected protoplasts, four (P1-801, HC-1937, HC-1937Δ15, and CP-8465) were able to produce systemic infections in tobacco plants. The symptoms produced ranged from very mild to symptoms as severe as those in wild-type infections (data not shown). Plants inoculated with the CP mutant, CP-8465, developed symptoms identical to those in plants inoculated with native viral RNA or pXBS7-derived RNA. These consisted of mild vein clearing of the first systemically infected leaf and pronounced vein mottling and blotching of younger leaves.

Plants inoculated with transcripts of mutant P1-801 exhibited a cyclical pattern of symptom development (data not shown). Initially, the first systemically infected leaves (leaf positions 4–5) developed very mild, barely discernible vein clearing. Younger leaves (positions 6–7) that developed approximately 20 days after inoculation exhibited only small sectors of pale green color and did not show vein clearing or mottling typical of a wild-type infection. Approximately 30 days after inoculation, however, newly appearing leaves (positions 9 and 10) began to show more pronounced vein clearing and by 45 days (leaf positions 12 to 15) typical TVMV-induced symptoms had appeared. As the P1-801-infected plants became

larger, they exhibited another cycle of leaves with mild symptoms followed by leaves with typical vein mottling.

Inoculation of plants with mutants HC-1937 and HC-1937Δ15 resulted in the development of symptoms that were strikingly different from typical TVMV-induced symptoms (data not shown). A delay of approximately 3 days in the appearance of symptoms was observed compared with plants inoculated with pXBS7-derived RNA. When symptoms did develop, they consisted of very pronounced vein clearing of the first systemically infected leaves (positions 5 and 6). With continued growth of the plants, however, symptoms on the newer leaves were sporadic, with vein clearing and blotching confined to areas around the outer edges of the leaves. At this stage, these leaves did not exhibit the vein mottling typical of plants infected with native viral RNA or pXBS7-derived RNA. By 25 days after inoculation, the newly expanding leaves (positions 9 and 10) were almost symptomless with only a few small areas of blotching around the outer edges of the leaf tips. By 45 days after inoculation, however, the young, developing leaves (positions 12 to 15) exhibited symptoms resembling those of a wild-type infection with vein mottling and blotching throughout the entire leaf. As with plants infected with mutant P1-801, plants inoculated with these two HCpro mutants exhibited a cyclical pattern of symptom expression.

Accumulation of virus in mutant-inoculated plants

ELISA and Northern blot analyses were performed to determine whether the altered patterns of symptom ex-

Inoculum	Relative virus titer[a] at leaf position						
	6	7	8	9	11	13	15
Experiment 1							
Mock	0.00	0.00	0.00	0.00	0.00	0.00	0.00
pXBS7	1.00	1.00	1.00	1.00	1.00	1.00	1.00
P1-801	1.20	0.59	0.67	0.90	1.15	1.19	1.00
HC-1937	0.07	0.03	0.03	0.00	0.03	ND[b]	ND
HC-1937Δ15	0.08	0.03	0.02	0.00	0.07	ND	ND
CD-8465	1.15	0.83	0.91	0.92	1.26	1.14	0.95

Inoculum	Relative virus titer at leaf position							
	6	7	8	9	11	12	14	15
Experiment 2								
Mock	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
pXBS7	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HC-1937	0.05	0.09	0.12	0.04	0.19	0.25	0.39	0.49
HC-1937Δ15	0.43	0.09	0.11	0.06	0.28	0.30	0.46	0.70

[a] The titer of virus in leaf extracts was estimated by double-antibody sandwich ELISA; each inoculum was applied to five plants and the mean titer of virus in leaves above the inoculated leaves was determined; values shown represent the ratio of the means of mutant to wild-type titer at each leaf position.

[b] ND, not determined.

pression in plants infected with mutants P1-801, HC-1937, and HC-1937Δ15 were accompanied by differences in the extent to which progeny virus accumulated in plants (Table 2 and Fig. 5). The levels of viral CP and RNA in leaves of plants inoculated with mutant P1-801 were not significantly different from those in plants infected with TVMV RNA or pXBS7-derived RNA (Table 2 and Figs. 5A and 5B) and suggest that the mild symptoms initially observed in the plants were not due to lower amounts of virus. Furthermore, the appearance of typical TVMV-like symptoms in leaves 45 days after inoculation (leaf positions 12–15) was not accompanied by an increase in virus accumulation.

In contrast, the amount of viral CP in leaves of plants inoculated with mutants HC-1937 and HC-1937Δ15 appeared to be correlated with the severity of symptom expression. Leaves with sporadic or no symptoms (positions 7–9) had much less viral CP than the corresponding leaves on plants infected with wild-type RNA (Table 2). Leaves exhibiting TVMV-like symptoms that appeared later (positions 12–15) contained CP although the levels were somewhat lower than those observed in plants inoculated with pXBS7-derived RNA. As with CP accumulation, the levels of viral RNA in plants infected with mutants HC-1937 and HC-1937Δ15 were consistently lower than RNA levels in plants inoculated with pXBS7 transcripts (Figs. 5A and 5B).

Analysis of plants inoculated with mutant CP-8465 indicated that throughout the course of the infection the amounts of viral CP and RNA were not significantly different from those detected in wild-type-infected plants (Table 2 and Figs. 5A and 5B). These results are consistent with the development of wild-type-like symptoms that were observed in plants inoculated with this mutant.

Stability of mutants in inoculated plants

Progeny viral RNA produced in plants inoculated with mutants P1-801, HC-1937, HC-1937Δ15, and CP-8465 was analyzed to verify that the 12-nt insertion was stably maintained in the TVMV genome. Since the insertions contain a unique XhoI site, only amplified fragments from progeny RNA that retained the insert would be digested by XhoI. Analysis of two selected leaves (leaf positions 6 and 15) revealed the presence of the XhoI site in progeny viral RNA from leaves of all mutant-infected plants and indicated that each of the four mutants was stable for at least the first 45 days after inoculation (data not shown). In addition, nucleotide sequencing of RT-PCR
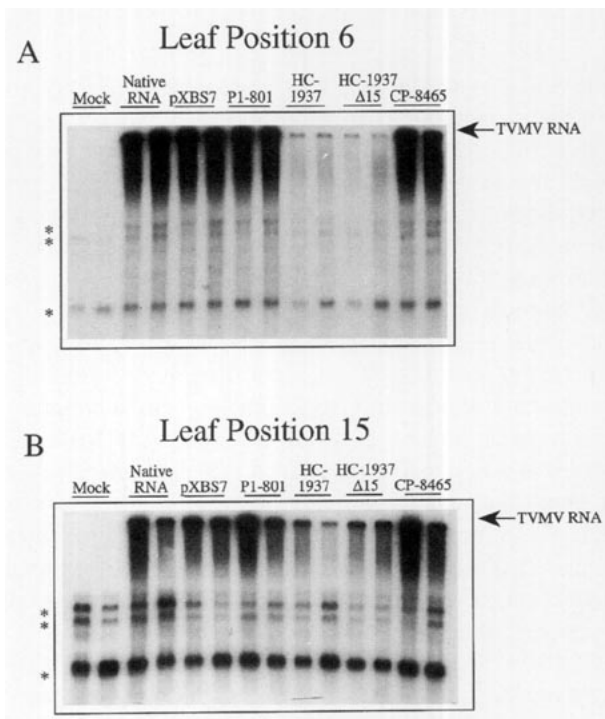

FIG. 5. Accumulation of TVMV RNA in plants inoculated with transcripts derived from wild-type and mutant cDNA plasmids. Total RNA was extracted from systemically infected leaves 15 (leaf position 6; A) and 45 (leaf position 15; B) days after inoculation. Two RNA samples isolated from individually inoculated plants of each treatment were analyzed. Samples equivalent to 2.5 mg of leaf tissue were electrophoresed on 0.8% agarose–formaldehyde gels, transferred to nylon membranes, and hybridized with a TVMV cRNA probe. Lanes containing samples from plants inoculated with native TVMV RNA or mock-inoculated are also shown. The arrow marks the position of TVMV genomic RNA. Asterisks mark the positions of ribosomal RNAs. Blots were exposed to X-ray film for 6 hr.

products generated from RNA isolated from plants inoculated with mutants HC-1937 and HC-1937Δ15 confirmed the presence of the original 12-nt insertion and indicated that no nucleotide changes had occurred in the region of the mutation (data not shown).

DISCUSSION

A genetic analysis of TVMV was undertaken to complement *in vitro* studies of the activities of potyviral proteins and to provide more information about the roles of these proteins in replication and pathogenicity. A collection of 19 mutants was constructed and they displayed a variety of phenotypes in inoculated plants and protoplasts (Fig. 2). Only one mutant (CP-8465) behaved like wild-type-like virus indicating that the insertion cartridge was itself not universally deleterious to the virus.

Replication-defective mutants

None of the mutants that contained inserts in the contiguous P3–CI–6K–NIa–NIb coding region of the TVMV genome was able to produce detectable amounts of progeny viral RNA in protoplasts. The proteins encoded by the CI, NIa, and NIb regions of the genome have previously been shown to possess activities or features assumed to be involved in the replication of potyviral RNA. The behavior of each of the three P3 mutants described here and the observed association of P3 with cylindrical inclusions in infected cells (Rodríguez-Cerezo *et al.*, 1993) suggest that this protein may also have such a function. Thus, as many as five proteins, encoded by a large internal segment of the potyviral genome, may have roles in replication.

The presence of 12-nt inserts in some of these parts of the TVMV genome may have resulted in the obstruction of important structural features of particular viral proteins. One of the insertions in the CI protein (mutant CI-4040) is three amino acid residues removed from one of the proposed sites of the NTP-binding domain (Gorbalenya and Koonin, 1989) and increases the distance between this and another such site. The other three insertions in this protein also disrupt conserved features (Koonin and Dolja, 1993; unpublished observations). The position of the insert in the so-called 6K region of mutant 6K-5516 is very close to the equivalent region in an insertion mutant of tobacco etch virus (TEV) that was reported to be nonviable (Restrepo-Hartwig and Carrington, 1994). The insert in mutant NIa-6652 occurs in a region corresponding to that shown by Dougherty *et al.* (1989) to encompass the catalytic triad in the NIa proteinase of TEV. Mutant NIb-7386 contains an insert in the region of NIb adjacent to the first (domain I) of the eight conserved domains that have been demonstrated in positive-strand RNA virus polymerases (Koonin, 1991).

Some of the other mutants may also have been replication-defective. The insertion in mutant P1-616 occurs just within the N-terminal boundary of a domain shown to be required for processing of the TEV P1-HCpro cleavage site (Verchot *et al.*, 1991). A defect in P1 proteinase activity would be expected to adversely affect the production of the HCpro protein and possibly the P3 protein and either of these consequences might result in impaired replication of viral RNA. Little or no progeny viral RNA was detected in protoplasts inoculated with mutants bearing insertions in the N- (HC-1500) and C-terminal (HC-2201) parts of the HCpro protein, respectively. Small changes (Atreya *et al.*, 1992; Atreya and Pirone, 1993) and large deletions (Dolja *et al.*, 1992) in the N-terminal regions of TVMV and TEV HCpro have been shown to result in significant reductions in accumulation of viral RNA and CP similar to those we observed (Fig. 4). The insert in mutant HC-2201 interrupts a proteolytically active domain that is highly conserved among potyviruses (Oh and Carrington, 1989) and it is likely that elimination of HCpro proteinase activity is responsible for the defect we observe in this mutant.

Movement-defective mutants

A cell-to-cell movement function has not yet been assigned to any of the potyviral proteins. However, it has recently been suggested that the CP of TEV may be involved in cell-to-cell movement (Dolja *et al.*, 1994). Of the mutants in our investigation, only HC-1378 replicated with reasonable efficiency in inoculated protoplasts, yet did not spread in inoculated leaves. Before declaring that HCpro may be a potyviral movement protein, however, we feel that it must be established that neither encapsidated nor unencapsidated mutant viral RNA moves from directly inoculated cells in a leaf to neighboring cells.

In our initial analysis, mutant 5′UTR-151Δ19 appeared to be a movement-defective mutant. However, transcripts of an independent clone of the construct did not display such a phenotype but rather had the characteristics of wild-type RNA (unpublished observations). Therefore, it is doubtful that the movement-defective phenotype was solely the result of the mutation in the 5′UTR. Sequence analysis and the construction of chimeric constructs of the two 5′UTR mutants may provide further insight into the viral protein(s) involved in cell-to-cell movement.

There have been some very modest indications that the P1 protein of TVMV may be a movement protein. Domier *et al.* (1987) reported a limited degree of amino acid sequence similarity between the TVMV P1 protein and the 30K movement protein of tobacco mosaic virus. The P1 of TVMV is capable of binding RNA in a relatively nonspecific manner (Brantley and Hunt, 1993), and several plant viral movement proteins have been shown to be RNA-binding proteins (Citovsky *et al.*, 1990, 1991; Schoumacher *et al.*, 1992; Osman *et al.*, 1993). In the present study, neither of the two mutants with insertions in the P1 protein exhibited a movement-defective pheno-

type. It is clear that further studies are necessary to conclusively demonstrate the role of TVMV proteins in cell-to-cell movement.

Mutants altered in symptom expression

Inoculation of plants with mutants of the P1 and HCpro proteins resulted in altered development of disease symptoms. Previous mutational analyses have demonstrated that mutations in both noncoding and coding regions of the potyviral genome can have profound effects on disease symptoms (Rodríguez-Cerezo et al., 1991; Atreya et al., 1992; Dolja et al., 1992; Atreya and Pirone, 1993). Thus, more than one region of the potyviral genome appears to be involved in symptom development, a phenomenon that seems to be a general one among plant viruses and that argues against the existence of viral proteins that are specific determinants of symptom production.

An interesting observation in plants infected with mutants P1-801, HC-1937, and HC-1937Δ15 was the cyclical nature of symptom expression. As plants developed, the symptoms progressed from mild or sporadic to more severe, and this pattern was then repeated as new leaves emerged. Why the symptom development should be cyclical is difficult to explain. Further characterization of the accumulation and distribution of virus and the development of symptoms in plants infected with these three mutants will be needed to better understand this novel and interesting phenomenon.

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